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Abstract: Anthropophilic mosquitoes are effective vectors of human disease because of their biting preferences. To find their host, these mosquitoes are guided by human odours, primarily produced by human skin bacteria. By analysing the skin bacterial and skin volatile profiles of humans, bonobos, chimpanzees, gorillas, lemurs and cows, we investigated whether primates that are more closely related to humans have a skin bacterial community and odour profile that is similar to that of humans. We then investigated whether this affected discrimination between humans and closely related primates by anthropophilic and zoophilic mosquitoes that search for hosts. Humans had a lower skin bacterial diversity than the other animals and their skin bacterial composition was more similar to that in other primates than it was to the skin bacteria of cows. Like the skin bacterial profiles, the volatile profiles of the animal groups were clearly different from each other. The volatile profiles of cows and lemurs were more closely related to the human profiles than expected. Human volatiles were indeed preferred above cow volatiles by anthropophilic mosquitoes and no preference was observed when tested against non-human primate odour, except for bonobo volatiles, which were preferred over human volatiles. Unravelling the differences between mosquito hosts and their effect on host selection is important for a better understanding of cross-species transmission of vector-borne diseases.

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RESEARCH ARTICLE

Do apes smell like humans? The role of skin bacteria and volatiles of primates in mosquito host selection

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ABSTRACT

Anthropophilic mosquitoes are effective vectors of human disease because of their biting preferences. To find their host, these mosquitoes are guided by human odours, primarily produced by human skin bacteria. By analysing the skin bacterial and skin volatile profiles of humans, bonobos, chimpanzees, gorillas, lemurs and cows, we investigated whether primates that are more closely related to humans have a skin bacterial community and odour profile that is similar to that of humans. We then investigated whether this affected discrimination between humans and closely related primates by anthropophilic and zoophilic mosquitoes that search for hosts. Humans had a lower skin bacterial diversity than the other animals and their skin bacterial composition was more similar to that in other primates than it was to the skin bacteria of cows. Like the skin bacterial profiles, the volatile profiles of the animal groups were clearly different from each other. The volatile profiles of cows and lemurs were more closely related to the human profiles than expected. Human volatiles were indeed preferred above cow volatiles by anthropophilic mosquitoes and no preference was observed when tested against non-human primate odour, except for bonobo volatiles, which were preferred over human volatiles. Unravelling the differences between mosquito hosts and their effect on host selection is important for a better understanding of cross-species transmission of vector-borne diseases.

KEY WORDS: Host preference, Mosquitoes, Primates, Vector diseases, Zoophilic, Apes

INTRODUCTION

Non-human primates often serve as reservoirs of human disease. The yellow fever virus (Ellis and Barrett, 2008), chikungunya virus (Labadie et al., 2010; McCrae et al., 1971) and malaria-causing *Plasmodium knowlesi* parasites (Cox-Singh et al., 2008; Kevin, 2009) are examples of pathogens that originate from non-human primates and are directly infectious to humans. Non-human primate habitat loss and closer interactions with humans is likely to lead to more cross-species transmission of pathogens (Isabirye-Basuta and Lwanga, 2008).

Malaria is caused by parasites of the genus *Plasmodium*, which are transmitted by mosquitoes. A large diversity of *Plasmodium* parasites exist that infect a wide range of vertebrate hosts. However, only five species of *Plasmodium* are known to cause malaria in humans, and these are all vectored by members of the mosquito genus *Anopheles*. *Plasmodium* species are largely host-specific (Liu et al., 2010), and the most common and lethal human malaria parasite *Plasmodium falciparum* has only occasionally been found to infect non-human primates in captivity (Duval et al., 2010; Pacheco et al., 2013). Parasites identical or very closely related to human *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* have been found in howler monkeys (Lalremruata et al., 2015), bonobos (Prugnolle et al., 2010), chimpanzees (Duval et al., 2010; Liu et al., 2010) and gorillas (Duval et al., 2010; Liu et al., 2010; Prugnolle et al., 2010), indicating their potential for cross-species transmission. Zoonotic transmission of *Plasmodium knowlesi* from monkeys to humans is known to occur frequently. This simian *Plasmodium* parasite was long known to be able to infect humans, but only when molecular techniques were employed did it become apparent that *P. knowlesi* infections are common in human populations in Malaysia (Cox-Singh et al., 2008; Kevin, 2009). This cross-species transmission may increase human infections and make control more difficult.

Host selection by mosquitoes drives the transmission of pathogens between humans; highly anthropophilic mosquitoes are often vectors of important human diseases (Takken and Verhulst, 2013). *Anopheles coluzzii* (formerly known as *Anopheles gambiae sensu stricto* molecular form M), for example, is an important malaria vector, and *Aedes aegypti* is an important vector of yellow fever and dengue; both have a restricted anthropophilic host range (McBride, 2016; Takken and Verhulst, 2013). Volatiles released by the human skin provide essential cues that guide these mosquitoes to their human host (Olanga et al., 2010). Skin bacteria play an important role in the production of these volatiles. *In vitro* studies have shown that skin bacterial volatiles attract *An. coluzzii* and *Ae. aegypti* (Verhulst et al., 2009; Zhang et al., 2015), and a study with 48 human individuals showed a correlation between human attractiveness to *An. coluzzii* and the composition of the human skin microbiota (Verhulst et al., 2010, 2011).

Skin glands play an important role in body odour production and are differentially distributed between humans and non-human primates (Smallegange et al., 2011). Even the closely related chimpanzees and gorillas have many fewer eccrine sweat glands than humans, whereas they have a higher abundance of apocrine glands (Smallegange et al., 2011). Only one study has characterized the skin microbiota of non-human primates in detail and showed that humans have a lower skin bacterial diversity (Council et al., 2016), probably because of personal hygiene and the lack of hairs. Interestingly, the apes tested in this study by Council et al. (2016), including humans, had a higher relative abundance of the family

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Staphylococcaceae when compared with monkeys. Both *Staphylococcus* and *Corynebacterium* species have been shown to produce volatiles that attract anthropophilic mosquitoes (Verhulst et al., 2010; Zhang et al., 2015), and individuals that are highly attractive to the anthropophilic malaria vector *An. coluzzii* have a higher abundance of *Staphylococcus* spp. on their skin (Verhulst et al., 2011).

The host preference of mosquitoes drives the transmission of mosquito-borne pathogens between humans; however, little is known about the role of host preference in cross-species transmission of pathogens. It remains to be investigated if anthropophilic mosquitoes are also attracted to primates closely related to humans and if the volatiles produced by *Staphylococcus* spp. or other skin bacteria play a role. In addition, it is unknown how the body odour profiles of non-human primates compare to that of humans, and if the similarities or differences are mediated by their skin microbiota (Smallegange et al., 2011). We hypothesize that primates that are more closely related to humans have a skin bacterial and odour profile that is more similar to that of humans and, therefore, that anthropophilic mosquitoes will not discriminate between humans and closely related primates when searching for their host. To address this hypothesis, we investigated the skin bacterial and volatile profile of five different primate species, including humans. In addition, we tested the attractiveness of the volatile samples to anthropophilic *An. coluzzii* and zoophilic *Anopheles quadriannulatus* mosquitoes (Athrey et al., 2017; Pates et al., 2014; Takken and Knols, 1999). As an external reference, we included cow skin bacterial and volatile samples and tested the volatiles for attractiveness to the two mosquito species.

MATERIALS AND METHODS

Mosquitoes

Anopheles coluzzii (Coetzee et al. 2013) from Suakoko, Liberia and *Anopheles quadriannulatus* Theobold 1911 from Sangwe, Zimbabwe were cultured at the Laboratory of Entomology of Wageningen University & Research, The Netherlands as previously described (Spitzen et al., 2013). The *An. coluzzii* mosquitoes were fed on human blood (Sanquin Blood Supply Foundation, Nijmegen, The Netherlands) and the *An. quadriannulatus* mosquitoes on bovine blood (Carus, Wageningen, The Netherlands). During feeding, a sock that released either human (*An. coluzzii*) or cow odours (*An. quadriannulatus*), was wrapped around the feeding membrane (Verhulst et al., 2013b).

Subjects

Between eight and nineteen skin bacterial and volatile samples were taken from each group of humans (*Homo sapiens*), western lowland gorillas (*Gorilla gorilla*), bonobos (*Pan paniscus*), chimpanzees (*Pan troglodytes*), ring-tailed lemurs (*Lemur catta*) and cows (*Bos taurus*) (sample overview Table 1). Non-human primate samples were taken from both males and females; the bonobo, gorilla and lemur samples were taken in Dutch zoos and the chimpanzee samples at a Dutch zoo and the Tchimpounga Rehabilitation Centre in The Republic of Congo (Table 1). Cows were free from antibiotics and sampled at two locations in The Netherlands (Table 1). Human subjects were all male, Caucasian and non-smoking. They were requested to refrain from drinking alcohol, eating garlic, onions or spicy food, taking a shower, using soap or perfumed cosmetics on the day before and during the 24 h of sampling (Verhulst et al., 2011). The individuals were free from chronic illnesses and did not use any medication on a regular basis.

Skin microbiota

Collection

Bacterial samples were taken with dual-tip cotton swabs (BD BBL™ CultureSwab™ EZ II, Becton Dickinson) that were rubbed 10 times over ±10 cm^2 of the upper forearm skin (in the same place as odours were collected) of the different animal species. Cow samples were taken from the skin above the femur of the hind leg. After sampling, the tips of each swab were cut off and stored individually in Eppendorf tubes at −20°C until DNA extraction.

DNA extraction, amplification and sequencing

DNA was extracted from one of the tips of each dual-tip swab using the PowerSoil® DNA Isolation kit (Mo Bio Laboratories). The cotton tips were transferred to the bead tubes included in the kit with 60 µl of kit solution C1. Next, the tubes were incubated at 65°C for 10 min and thereafter horizontally shaken in the Mo Bio vortex adapter at maximum speed for 15 min (Costello et al., 2009). The remaining steps were executed as directed by the manufacturer. Isolated DNA was PCR amplified with 16S ribosomal RNA (rRNA) targeted primers 515f and 806r (Apprill et al., 2015). Each 25 µl PCR reaction contained 9.5 µl of MO BIO PCR Water (certified DNA-free), 12.5 µl of QuantaBio AccuStart II PCR ToughMix, 1 µl forward and reverse primers (5 µmol l^{-1} concentration) and 1 µl of template DNA. The conditions for PCR were as follows: denaturation at 94°C for 3 min, 35 cycles at 94°C for 45 s, 50°C for 60 s and 72°C for 90 s; with a final extension of 10 min at 72°C.

Table 1. Overview of the sample location and number of animals from which the skin bacteria and skin volatiles were taken

Species	Common name	Sample location	Sex	No. of bacterial samples	No. of volatile samples	No. of pads in olfactometer
Homo sapiens	Human	The Netherlands	Male	15	12	2.5
Pan paniscus	Bonobo	Apenheul, Apeldoorn, The Netherlands	Male (7), Female (3)	10	10	5
Pan troglodytes	Common chimpanzee	Burgers' Zoo, Arnhem, The Netherlands & Tchimpounga Chimpanzee Rehabilitation Centre, Republic of Congo	Male (12), Female (4), Unknown (3)	19	13	0.5
Gorilla	Western lowland gorilla	Gaia Zoo, Kerkrade, The Netherlands	Male (6), Female (9)	15	14	0.5
Lemur catta	Ring-tailed lemur	De Apenheul, Apeldoorn & Ouwehands Dierenpark, Rhenen, The Netherlands	Male (3), Female (10), Unknown (2)	15	8	5
Bos taurus	Cow	Carus, Wageningen & Veld en Beek, Doorwerth, The Netherlands	Female	15	12	0.5

Volatile samples were used for both volatile analysis and mosquito behavioural experiments (different samples from the same individual). The number of pads used in the olfactometer experiments in each test was always composed of pieces of pads from four different individuals to exclude individual effects.

Amplicons were quantified using PicoGreen (Invitrogen) and a plate reader (Infinite® 200 PRO, Tecan). Once quantified, volumes of each of the products were pooled so that each amplicon was represented in equimolar amounts. Pools were cleaned up with AMPure XP Beads (Beckman Coulter), and then quantified using a fluorometer (Qubit, Invitrogen). After quantification, the molarity of the pool was determined and diluted to 2 nmol l^{-1}, denatured and then diluted to a final concentration of 6.75 pM with a 10% PhiX spike. Amplicons were sequenced at Argonne National Laboratory, Lemont, USA, on a 151 bp×12 bp×151 bp MiSeq (Caporaso et al., 2012).

Sequence analysis

Initial analysis of raw 16S rRNA gene sequencing data was performed using NG-Tax pipeline (Ramiro-Garcia et al., 2016). Operational taxonomic units (OTUs) were defined using an open reference approach, and taxonomy was assigned using a SILVA v123 16S rRNA gene reference database (Quast et al., 2012). R environment (R version 3.4.1) was used for downstream data manipulations, statistical analysis and visualization. Samples with a low number of reads (<1500) were removed from the data set, as were outliers. In addition, reads assigned as chloroplast, mitochondria or not assigned at all were removed. Finally, OTUs that were encountered in fewer than three samples or had fewer than two reads were removed. Alpha diversity indices were calculated on a rarefied OTU matrix. Samples were rarefied at 1527 reads depth as implemented in the phyloseq package. Number of observed species and Inverted Simpson index were calculated using the 'estimate_richness' function from the same package. Phylogenetic diversity was calculated using 'pd' function from the picante package, with phylogenetic tree rooted at the middle point with the 'midpoint' function from the phangorn package. Bray dissimilarity matrix was constricted using the 'vegdist' function from the vegan package to investigate the beta diversity of microbial communities. Non-metric multidimensional scaling (NMDS) was performed as implemented in the same package, for visualization of beta diversity. Statistical significance in beta diversity variations between selected groups of samples and strength of the model were determined by the function 'adonis' as implemented in the vegan package.

Skin volatiles

Collection

For all animals, except human volunteers, skin volatiles were collected on two cotton pads that were rubbed 10 times over ±10 cm^2 of the lower arm and/or hand. Human volunteers were asked to wear the two pads for 24 h on the lower arm, covered with an aluminium foil layer and an island plaster (Verhulst et al., 2016). After sampling, the pads were stored at −20°C in 10 ml glass vials. Vials and pads were cleaned before use as previously described (Verhulst et al., 2016).

Volatile analysis

Small samples of 13.18±0.07 mg (mean±s.e.m.) were taken of each cotton pad, and transferred to an empty glass tube (Markes International Ltd, Llantrisant, UK) for direct desorption; the tube was then placed in an auto-sampler desorption unit (Ultra 50:50 TD, Markes International) (Verhulst et al., 2016). Desorption from the cotton samples, separation, detection and identification of volatiles were carried out as described previously by Verhulst et al. (2016) using gas chromatography–mass spectroscopy (GC–MS) with minor modification; thermal desorption was carried out at 150°C for 20 min while re-collecting the volatiles in a thermally cooled universal solvent trap (Unity, Markes, Llantrisant, UK) at 0°C. Injected at 5:1 ratio, further separation of volatiles was done using ZB-5MSi analytical column [30 m×0.25 mm I.D.×1.00 m F.T. with a 10 m built-in guard column (Phenomenex, Torrance, CA, USA)]. The GC initial temperature was 40°C for 2 min and was immediately raised at 6°C min^{-1} to a final temperature of 280°C that was maintained for 4 min. Volatiles detected in the samples, excluding those in the control clean cotton pads, were batch-processed in Xcalibur (Version 2.07, Thermo Scientific, USA) for relative quantification as described previously (Verhulst et al., 2013a).

Statistics on volatile profiles

Peak areas of identified volatiles in the chromatograms were first corrected for the weight of the cotton pad section used. Further analyses were performed as described previously (Verhulst et al., 2013a, 2016). In short, peak areas were log transformed, mean centred and scaled to unit variance before principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA, SIMCA-P 12.0, Umetrics, Sweden). PCA and PLS-DA were used for group separation and pattern recognition among the different animals, based on their body odour profiles. Creation of a loading plot allowed visualization of the correlation of volatiles to the different animal odour profiles (Eriksson et al., 2006; Verhulst et al., 2013a). The number of significant PLS-DA components was determined by cross-validation (Eriksson et al., 2006). Analysis was performed with SPSS (2016, version 24, IBM).

Mosquito host preference

The attraction of the anthropophilic *An. coluzzii* mosquito and the more zoophilic *An. quadriannulatus* to volatiles collected from the different animal species, was tested in a dual-choice olfactometer in a climate controlled room (28°C, RH 60%, 1 lx; Verhulst et al., 2010). To correct for the quantity of the (skin) volatiles on the pads, parts of the cotton pads with volatiles from the different animals were pooled for each animal species. Correction was done based on the GC-MS chromatograms. For each species, the average total ion count of the chromatograms was calculated to determine the ratio of volatile abundance between the different host species. Pads of four individuals of each species were cut into eight pieces each. Depending on the ratio calculated, between 0.125 and 1.25 pads (Table 1) of each individual of a species were combined in a bundle and supplemented with clean pads to five pads in total. Each set of pads from each animal species was then tested in the olfactometer against the set of human odours to determine the preference of females of the two mosquito species for the different hosts.

The olfactometer consisted of a polycarbonate flight chamber of 1.60×0.66×0.43 m, which contained two small ports on the upwind side from which odours could be released and mosquitoes trapped. The mosquitoes were released from a release cage on the downwind side (Verhulst et al., 2010). The odours were dispersed through the flight chamber by pressurized air that was charcoal filtered, heated (30°C) and humidified (>80% RH). The airflow was led through the two trapping devices towards the side of the mosquito release point with a velocity of 0.15–0.21 m s^{-1}. In addition, CO_2 was released from the two trapping devices at 170 ml min^{-1}. Groups of 20–30 female mosquitoes were randomly collected 14–18 h before the start of the experiments and placed in a cylindrical release cage (8×10 cm). *Anopheles coluzzii* mosquitoes were starved overnight by removing sugar water, but they still had access to tap water via a piece of wet cotton wool. *Anopheles quadriannulatus* mosquitoes were provided with sugar water until the experiments started. For all

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experiments, 4- to 12-day-old female mosquitoes that had not received a blood meal were used. Mosquitoes were only used once.

The experiments took place during the last four hours of the scotophase. In each trial, the test odours were released in the airstream before releasing the mosquitoes from the cage. After 15 min, the females that were trapped were counted. Each test started with new odour samples, new mosquitoes and clean trapping devices. Experiments were repeated six times and the samples were alternated between ports to minimize positional effects.

Statistics on mosquito preference

Data were analysed with a generalized linear model (GLM; binomial distribution, logit link function, dispersion estimated) to investigate differences in attractiveness between the standard human odour and animal odour tested. The relative attractiveness was expressed as the number of mosquitoes caught in one trapping device divided by the total number of mosquitoes trapped in the two trapping devices together (binomial total; Qiu et al., 2006). The relative attractiveness was used to investigate whether the human volatiles collected more mosquitoes than the animal volatiles, when tested directly against each other (Qiu et al., 2006; Verhulst et al., 2011). The 95% confidence interval (CI) derived from the GLM of the predicted proportion of mosquitoes choosing the different odour samples was used to assess whether mosquito choice differed significantly from a 50:50 distribution (Robinson et al., 2018). The effects of position of treatment in the olfactometer, temperature, humidity and their interactions were fitted as parameters in the model when significant and models were compared using the corrected Akaike's information criterion (AICC). Analyses were performed with SPSS.

Ethical clearance

Informed consent was acquired from all human subjects prior to participation. The study was in accordance with the experimental protocol that was reviewed by the Medical Ethical Reviewing Committee of Wageningen University (METC-WU). The METC-WU concluded that the study did not fall within the remit of the 'Medical Research Involving Human Subjects Act', which means that the researchers are lawfully not obliged to obtain ethical approval from a recognized medical research ethics committee for this particular research. Taking skin bacterial and volatile samples from animals does not cause any discomfort and therefore does not require ethical clearance under the Dutch law on animal experimentation. Sample collection in The Republic of Congo was approved by the Ministry of Forest Economy and Sustainable Development under permit no. 071.

RESULTS

Skin microbiota

A total of 1487 unique OTUs belonging to 19 prokaryotic phyla were obtained. More than 90% of all OTUs could be classified within the phyla Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes, with distributions similar among the animal species, except lemurs that had a lower proportion of Firmicutes (Fig. 1 and Fig. S1). Alpha diversity as measured by either the number of different OTUs, inverted Simpson index or phylogenetic diversity

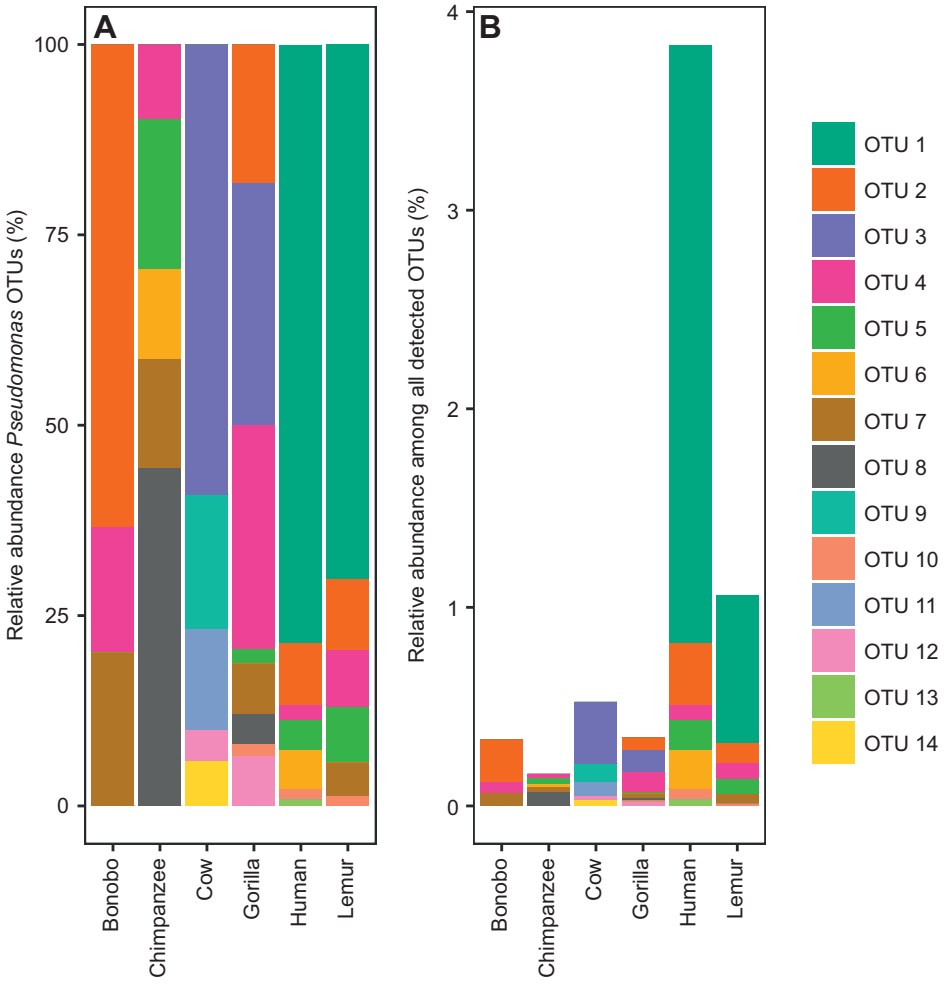

Fig. 1. Compositional bar charts of the relative abundance of the 14 most important *Pseudomonas* OTUs across investigated samples. (A) Relative abundance of *Pseudomonas* OTUs within species. (B) Relative abundance of *Pseudomonas* OTUs among all species.

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index, was lowest in the human samples and significantly different from the high bacterial alpha diversity found on the cow skin (Fig. 2A). In addition, the phylogenetic diversity index of the bonobo skin bacterial samples was also higher than that of the human samples (Fig. 2A).

Previous studies have shown that volatiles from *in vitro* grown *Staphylococcus epidermidis* attract mosquitoes, whereas *Pseudomonas aeruginosa* volatiles do not (Verhulst et al., 2010). In addition, the relative abundance of *Staphylococcus* spp. on the skin of 48 humans was positively correlated with the attraction of the malaria mosquito *An. coluzzii*, while the effect was opposite for *Pseudomonas* spp. (Verhulst et al., 2011). Therefore, the relative abundance of these two genera was determined in this study. Humans had the highest relative abundance of *Staphylococcus* and *Pseudomonas* bacteria as determined by the read counts (37.4±22.1% and 3.8±2.7%, respectively; mean±s.d.; Fig. 2B). The relative abundance of *Staphylococcus* on the skin of humans, chimpanzees and gorillas was significantly higher than the relative abundance on cow skin, which was only 1.1±1% (Wilcoxon, $P<0.05$, Fig. 2B). The relative abundance of *Pseudomonas* spp. was also highest on human skin and significantly higher than on the skins of chimpanzee and gorilla ($P<0.001$ and $P=0.015$, respectively; Fig. 2B).

NMDS ordination of genus-level microbial composition of different animals clearly grouped the animals based on their skin microbiota ($r^2=0.47$, $P=0.001$, Fig. 2C). As expected, the skin

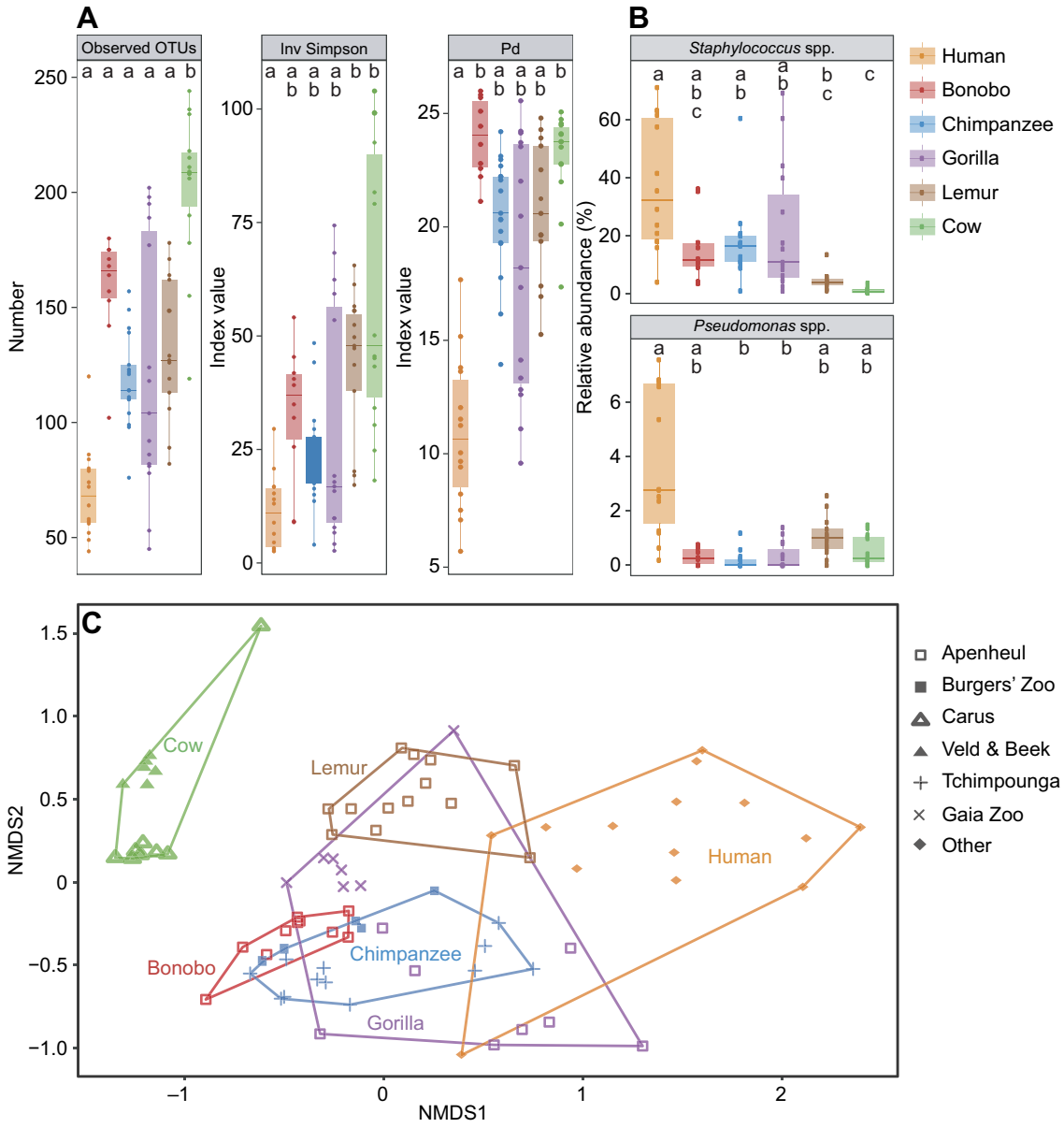

Fig. 2. Bacterial composition of skin in different animals. For sample overview, see Table 1. (A) Boxplot of alpha diversity variation between different species. Number of observed OTUs, inverted Simpson index (Inv Simpson) and phylogenetic diversity index (Pd) are plotted separately. Letters above bars indicate significant differences in diversity between groups as assessed by Wilcoxon test followed by pairwise comparison with Bonferroni correction (plot A+B). (B) Boxplot of the relative abundance (for each individual: read counts within a bacterial genus/total read count) of two bacterial genera that were previously linked to human attractiveness to mosquitoes (Busula et al., 2017; Verhulst et al., 2011). (C) Nonmetric multidimensional scaling (NMDS) ordination of microbial genera of different animals using Bray–Curtis distances for dissimilarity matrix. Relative abundance of microbial genera was used as input data for construction of dissimilarity matrix. Adonis confirmed statistical significance of grouping ($r^2=0.47$, $P=0.001$).

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bacterial composition of cows was different from that of the five primate species sampled, including humans. The non-human primate skin bacterial profiles overlapped with the gorilla skin bacterial profiles, which were more variable (Fig. 2C). The skin bacterial profiles of lemurs were not more distinct from humans than the profiles of the other primates, although lemurs belong to a different phylogenetic suborder.

Skin volatiles

Odourless cotton pads with collected skin volatiles were analysed by GC-MS (Verhulst et al., 2013a). Thirty compounds were more abundant in the chromatograms of the animal samples than in the chromatograms of the control pads (Fig. 3 and Fig. S2). A PCA resulted in three significant principal components of the data that explained 51% of the variation in the data and showed a clear

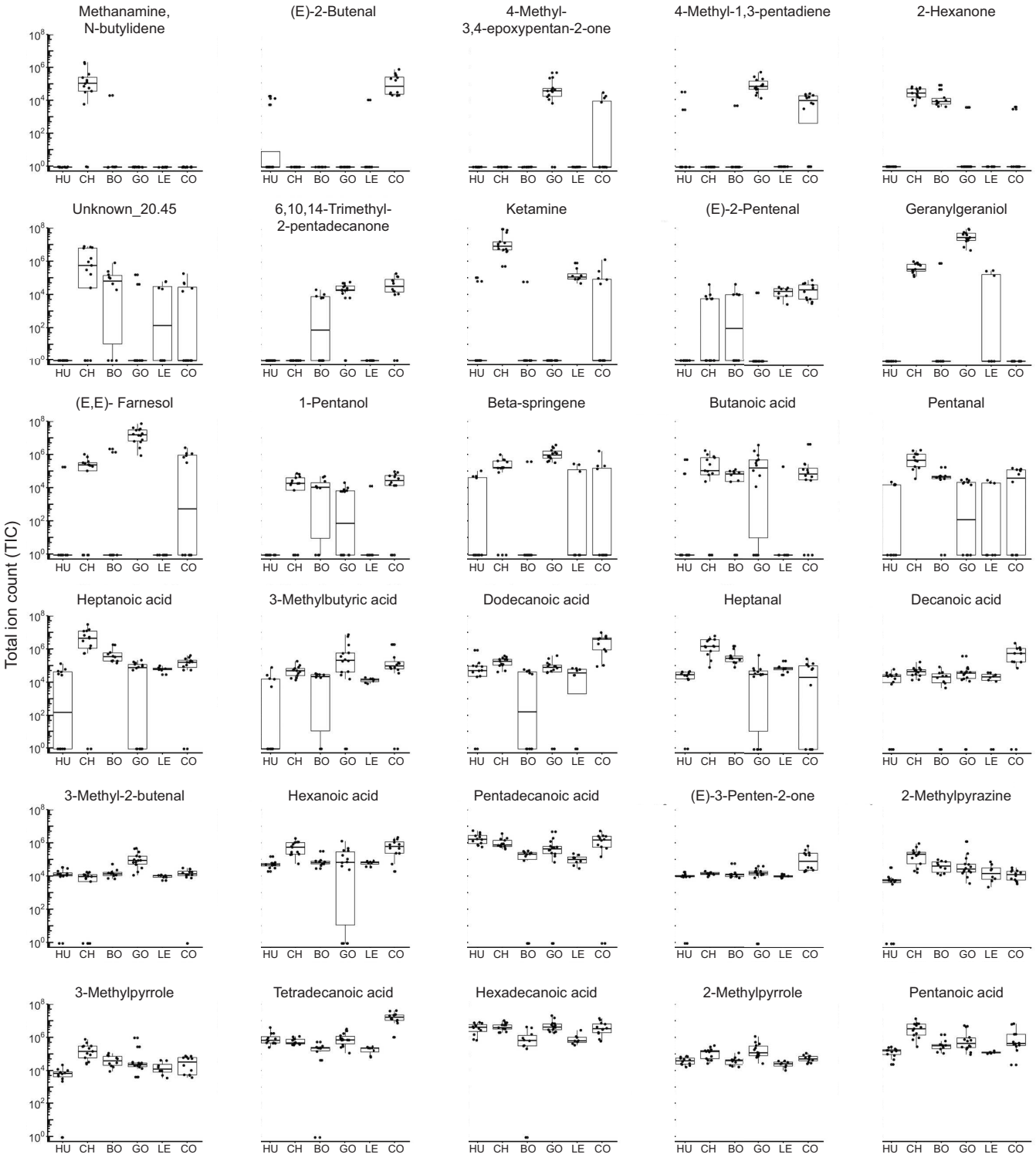

Fig. 3. Abundance (log total ion count) of volatile compounds identified in the skin volatile profiles of the different animals. Number of samples per animal species can be found in Table 1. HU, human; CH, chimpanzee; BO, bonobo; GO, gorilla; LE, lemur; CO, cow.

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separation of the volatile profiles of the different animals [$R^2(x)$=0.510, Q^2=0.293, Fig. S3). PLS-DA was used to visualize the differences in the volatile profiles and identify the volatiles that were most important in the separation of the treatments (Fig. 4). The PLS-DA extracted six significant axes that together explained 65.9% of the variation in the data [$R^2(x)$=0.659, $R^2(y)$=0.823, Q^2=0.75, Fig. 4]. The five volatile compounds that were most influential for the separation of the volatile profiles of the different animals were (*E*)-2-pentenal, ketamine, (*E*)-2-butenal, geranylgeraniol and 2-hexanone with VIPs (variable importance in the projection values) larger than 1.25.

Both PCA and PLS-DA showed that the human odour profiles were more closely related to the lemur and cow profiles than the profiles of the other animals. None of the compounds identified in this study was specific for one animal species, although the PLS-DA revealed some compounds that were more associated with one animal species than others (Figs 3,4 and Fig. S2).

Mosquito host preference

The choice of the anthropophilic *An. coluzzii* was significantly affected by the combination of volatiles offered (GLM, P=0.026, including the position of the treatment as covariate). *Anopheles coluzzii* had a significant preference for the human volatiles when tested against cow volatiles. However, when human volatiles were tested against bonobo volatiles, the bonobo volatiles were preferred (GLM, 95% CI, Fig. 5). No preference of *An. coluzzii* was detected when human volatiles were tested against chimpanzee, gorilla or lemur odour. The preference of the more zoophilic *An. quadriannulatus* was also significantly affected by the combination of volatiles offered (GLM, P<0.001); however, only the test between human volatiles and gorilla volatiles resulted in a significant divergence from a 50:50 distribution, with gorilla volatiles being more attractive (GLM, 95% CI, Fig. 5). No significant differences were found between the total number of mosquitoes that were trapped in both trapping devices for both

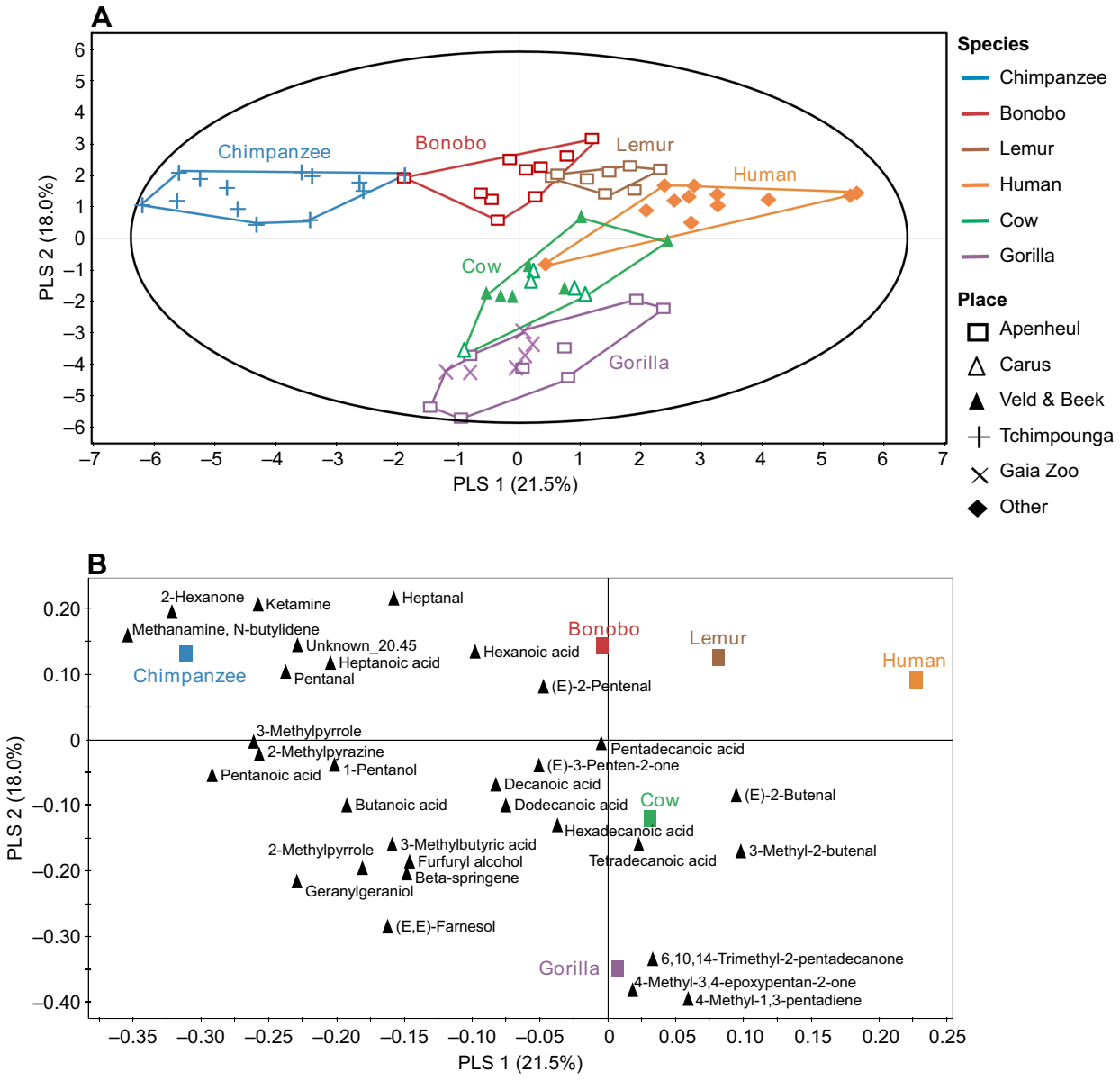

Fig. 4. Composition of blends of skin volatiles from different animals. Projection to latent structures discriminant analysis (PLS-DA) score plot (A) and loading plot (B) based on the amounts (log) of 30 volatile compounds from the skin emanations of different animals (Table 1). Volatiles closer to the animal in the plot (B) are more correlated to either group of animals. The ellipse in A defines the Hotelling's T^2 confidence region (95%).

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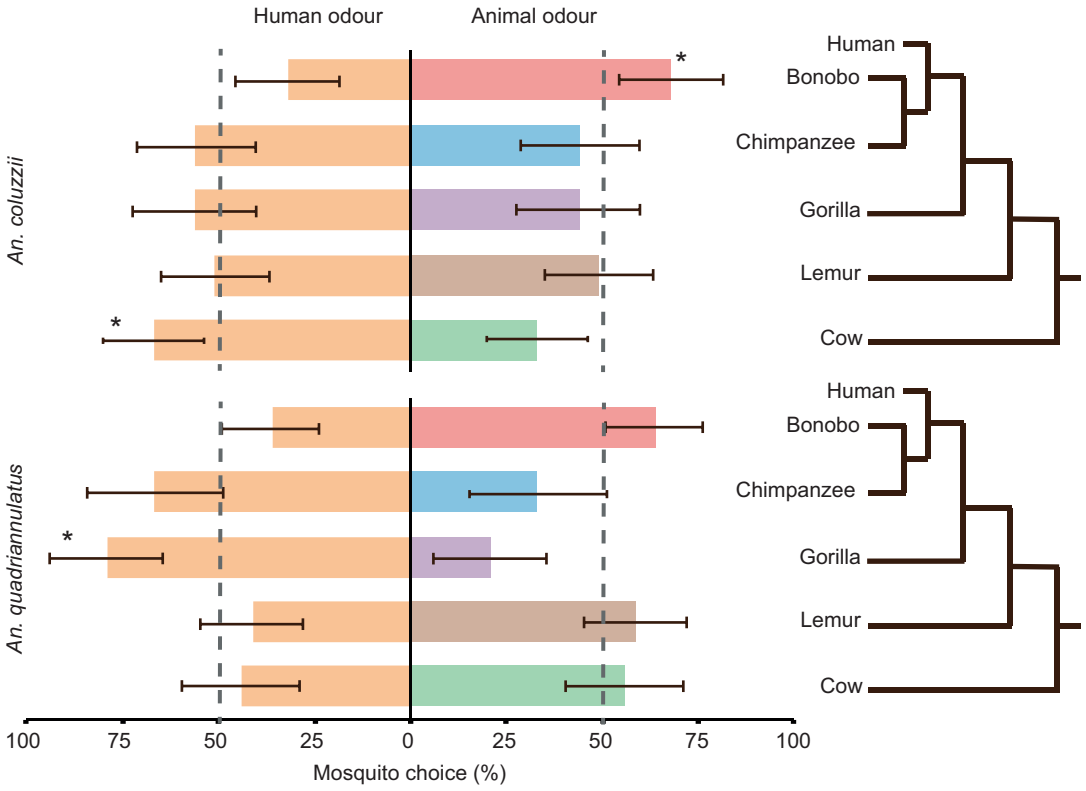

Fig. 5. Response of anthropophilic *An. coluzzii* and zoophilic *An. quadriannulatus* to human and animal odours. Volatiles were released in a dual-choice olfactometer from cotton pads of which the quantity was corrected for the volatile intensity, based on the total ion count (Table 1). For each replicate (6×) 30 *An. coluzzii* or 20 *An. quadriannulatus* were tested, see Table S1 for release and response rates. Predicted mean percentage caught and confidence intervals (GLM) are presented from six replicates. Colours indicate the different animal species. *Treatments that were different from a 50:50 distribution, determined by 95% two-sided confidence intervals. Phylogenetic tree indicates relatedness, but is not to scale.

mosquito species (GLM, *An. coluzzii* P=0.132, *An. quadriannulatus* P=0.053, Table S1).

DISCUSSION

The hypothesis that the skin microbial profile of humans is more similar to those of other primates was substantiated by the fact that the cow skin microbiota was clearly different from the primate microbial communities. The diversity of the human skin microbial community was much lower than the skin microbial diversity of the other animals tested, which is probably related to the lack of hairs on the human body and personal hygiene practices (Council et al., 2016).

Council et al. (2016) showed that the skin microbial profiles of apes (chimpanzees and gorillas) are more closely related to profiles in humans than those of monkeys (rhesus macaques and baboons). Therefore, it was expected that the microbial profiles of the apes tested in this study would be more closely related to humans than to the profiles of lemurs; however, this was not the case (Fig. 2C). In concordance with the study of Council et al. (2016), the relative abundance of *Staphylococcus* spp. was especially high in the human skin profiles. *Staphylococcus* spp. were less abundant in the ape samples and even lower abundances were found in the non-ape samples investigated here as well as in the study of Council et al. (2016). The *Staphylococcus* spp. distribution on the human skin is associated with the abundance of eccrine glands and is therefore predominant on the human feet and hands (Smallegange et al., 2011; Wilson, 2008). Non-human primates have a much lower abundance of eccrine glands, although higher than most other animals (Smallegange et al., 2011). These differences in abundance of eccrine glands could consequently explain the abundance of *Staphylococcus* spp. found in the different animal bacterial samples in this study. Previous studies have shown that volatiles from *in vitro* grown *Staphylococcus epidermidis* attract anthropophilic mosquitoes (Verhulst et al., 2009; Zhang et al., 2015), and that the relative abundance of *Staphylococcus* spp. on the skin is positively correlated with the attraction to *An. coluzzii* (Verhulst et al., 2011).

Also unexpected was the high relative abundance of *Pseudomonas* spp. in the human samples compared with the samples from the other animals, although the total count of *Pseudomonas* spp. may still be lower. *Pseudomonas* spp. are commonly found on the human skin (Grice et al., 2009, 2008; Wilson, 2008), but are also abundant in soil samples (Cavalca et al., 2015; Feltman et al., 2001). Therefore, the relative abundance of *Pseudomonas* spp. was expected to be higher in the cow bacterial communities as their skin is in frequent contact with soil. This discrepancy may be explained by a difference between cow and human in total count of *Pseudomonas* spp., but also by differences that occur at a species rather than a genus level. A more detailed analysis of the *Pseudomonas* genus revealed a distinct composition and relative weight differences between OTUs that comprise the *Pseudomonas* genus within a species-specific host group (Fig. 3 and Fig. S1). Metabolic conversions may differ between bacterial species, thereby leading to a difference in odour production (Ara et al., 2006; James et al., 2004) and possibly attraction to mosquitoes.

There was a high intraspecific variation in the volatile profiles, both in quantity and in composition, which could be true inter-individual variation or variation caused by the collection method. All methods used to collect volatiles have their limitations, and cotton pads, for example, do not only collect headspace volatiles, but also semi-volatiles (Birkemeyer et al., 2016). However, this was the only method we could apply quickly to non-anaesthetized primates that move and often try to grab the materials. In addition, an advantage of the cotton pads was that they consisted of similar material as the cotton bacterial swabs and could be used in both the volatile analysis and the behavioural experiments without any extraction or modification. Volatiles like ammonia and lactic acid are difficult to collect on most adsorbents and were not included in this study, although they are known to be common on the human skin. Carboxylic acids have also been described as human-specific volatiles and attractants for anthropophilic mosquitoes (Nicolaides et al., 1968; Smallegange et al., 2011). We identified several carboxylic acids of which some are known mosquito attractants; however, they were not more abundant in the human samples than in the samples of the other animals (Fig. 3). In total, 30 volatile compounds could be identified that were significantly more abundant in the animal skin samples than in the control samples, and PCA and PLS-DA clearly distinguished the volatile patterns of the different animals (Fig. 4). Similarly to the bacterial profiles, the volatile profiles of lemurs were more closely related to the volatile profiles of humans than those of the other primates. Previous studies did not show a correlation between the skin microbiota of zoo animals and their caretakers (Council et al., 2016), which could have explained the relatedness of these profiles. In addition, there was no physical contact between the lemurs and humans in the zoos where these samples were taken.

While previous studies have shown correlations between the skin bacterial composition of humans and the odours they emit (Ara et al., 2006; Taylor et al., 2003; Xu et al., 2007), no clear correlations between the skin microbial and odour profiles of the different animals could be established in this study. There could be several explanations for these differences: (1) the volatiles detected are, at least partly, not of bacterial origin but directly released from the skin; (2) the cotton pads used as an adsorbent for the skin volatile and subsequent procedures did not result in the detection of the full volatile profile; (3) 16S rRNA gene amplicon sequencing does not discriminate between dead bacteria and the active fraction of the skin microbiota that contribute to volatile production. Meta-transcriptomics may reveal the underlying processes of odour production on the skin (Fredrich et al., 2013; Verhulst and Takken, 2015) and better explain the volatile profiles presented in this study. Sampling from different body parts may reveal intra-individual variation caused by differences in skin gland abundance (Stoddart, 1990; Wilson, 2008). Although previous studies have found that mosquitoes may select specific biting sites on the body (Braack et al., 2015; Dekker et al., 1998), a study with odour samples taken from the hand, foot and armpit did not reveal any differences in attraction to *An. coluzzii* (Verhulst et al., 2016).

It was hypothesized that anthropophilic mosquitoes would be more attracted to human volatiles than to cow volatiles but would not discriminate between human and non-human primate volatiles. This hypothesis was partly confirmed because human volatiles were indeed preferred over cow volatiles by *An. coluzzii*, and no preference was observed when human odour was tested against non-human primate odour, except for bonobo volatiles, which were preferred over the human volatiles (Fig. 5). In previous studies, the more zoophilic *An. quadriannulatus* mosquitoes preferred cow odours over human odours (Athrey et al., 2017; Dekker and Takken, 1998) or the mosquitoes did not seem to have a preference (Pates et al., 2005), as in this study. The higher proportion of *An. quadriannulatus* caught in the trapping devices with human odour when tested against gorilla odour may be caused by volatiles in the gorilla odour profile that reduce attractiveness or even repel *An. quadriannulatus*. This is supported by the low response of the mosquitoes in this dual-choice test compared with the other tests (Table S1). To examine this hypothesis, volatiles that were more abundant in the gorilla profiles (Fig. 3) would need to be tested in more detail.

Sampling of skin bacteria and skin volatiles from non-human primates is challenging and this will affect both the quantity and quality of the samples collected. Human males and not females were sampled, because the female menstrual cycle is known to influence their odour and probably also their skin bacterial profile (Havlíček et al., 2006) and attraction to mosquitoes (Roessler and Brown, 1964). Samples from the other primates were collected from both males and females, because the number of individuals that was available was limited and all cattle samples originated from cows. Therefore, some of the differences found between species in this study could also reflect the differences between sexes, although in general the differences between sexes were smaller than the differences between species (Fig. S5). Another limitation due to the work with non-human primates was the number of odour pads that were sampled per species, resulting in only six replicates for each behavioural comparison, while a higher number of replicates would increase the accuracy of the data.

In nature, host selection by mosquitoes does not depend only on the skin volatiles emitted by a certain host. For example, non-anthropophilic mosquitoes rely much more on the detection of carbon dioxide to find their host (Takken and Verhulst, 2013). Therefore, large hosts that emit more carbon dioxide, will be found more easily by such mosquitoes (Dekker and Takken, 1998). In addition, other cues such as colour, body heat, body mass and defensive behaviour may all have an impact. Especially in conditions with a low abundance of preferred hosts, host preference of mosquitoes is also characterized by high plasticity, resulting in the mosquitoes choosing other hosts (Takken and Verhulst, 2013). Little is known about the forest mosquitoes that could facilitate disease transmission between non-human primates and humans, and how important the role of host preference is in this transmission. Makanga and colleagues (2017) showed that *Anopheles* mosquitoes harbouring ape *Plasmodium* in their salivary glands were also biting humans, indicating that cross-species transmission may occur. However, the rate at which these mosquitoes bite non-human primates and humans still needs to be determined.

The results presented in this study show clear differences between either the skin bacterial or skin volatile profiles of the different animals. Although, no correlation was found between the degree of anthropophily of the mosquitoes tested and the skin microbial and skin volatile profiles of their host, the mosquitoes with different host preferences responded differentially to the animal odours tested. The high density of eccrine glands in human skin and the associated high abundance of *Staphylococcus* spp. may lead to the production of volatiles that specifically attract anthropophilic mosquitoes. The differences in host preferences of mosquito species would affect their role as a bridge vector of diseases. A better comprehension of the differences between potential hosts and how these influence host selection by disease vectors will lead to a better understanding of cross-species transmission of vector-borne diseases.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.V., W.T.; Methodology: N.V., A.U., B.W., T.V., H.S.; Formal analysis: N.V., A.U., B.W.; Investigation: J.M., T.V.; Data curation: A.U., B.W., J.M., T.V.; Writing - original draft: N.V., A.U., B.W.; Writing - review & editing: N.V., A.U., B.W., J.M., T.V., M.D., H.S., W.T.; Visualization: N.V., A.U.; Supervision: N.V., M.D., H.S., W.T.; Funding acquisition: N.V., M.D., W.T.

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Data availability
The sequence data generated during the current study are available in the European Nucleotide Archive repository (accession number PRJEB28608). All other data generated during the current study is available from the corresponding author on reasonable request.

Supplementary information
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